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Research report

Epidural resiniferatoxin induced prolonged regional analgesia to pain

Tamas Szabo [a], Zoltan Olah [b], Michael J. Iadarola [b], Peter M. Blumberg [a,*]

[a] *Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institute of Health, Bldg 37, Rm 3A01 9000 Rockville Pike, Bethesda, MD 20892, USA*

[b] *Neuronal Gene Expression Unit, NIDCR, National Institute of Health, Bethesda, MD 20892, USA*

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Abstract

Adequate treatment of cancer pain remains a significant clinical problem. To reduce side effects of treatment, intrathecal and epidural routes of administration have been used where appropriate to reduce the total dose of agent administered while achieving regional control. Resiniferatoxin (RTX), an ultrapotent capsaicin analog, gives long-term desensitization of nociception via C-fiber sensory neurons. We evaluate here the analgesic effect on rats of epidurally administered RTX, using latency of response to a thermal stimulus in unrestrained animals. Results were compared with those for systemically administered RTX. Vehicle or graded doses of RTX were injected subcutaneously (s.c.) or through an indwelling lumbar (L4) epidural catheter as a single dose. Both routes of application of RTX produced profound thermal analgesia, reaching a plateau within 4–6 h and showing no restoration of pain sensitivity over 7 days. Vehicle was without effect. For the epidural route, the effect was selective as expected for the targeted spinal cord region, whereas the subcutaneous administration of RTX had a generalized analgesic effect. At doses yielding a tripling of back paw withdrawal latency, epidural treatment was 25-fold more effective than the subcutaneous route of application. Consistent with the regional selectivity of the lumbar epidural route, the front paws showed no more effect than by systemic RTX treatment. Binding experiments with [^{3}H]RTX provided further evidence of the segmental desensitization induced by epidural RTX. We conclude that epidural administration of RTX at the lumbar spinal level produces profound, long-lasting, segmental analgesia to C-fiber mediated pain in the rat.

Keywords: Resiniferatoxin; Capsaicin; Vanilloid; VR1; Nociception

1. Introduction

Capsaicin, the pungent ingredient in hot chili peppers, has been widely used both in vivo and in vitro as a tool to study the function of primary sensory neurons in nociception [2,3,14]. Although there is no complete overlap with other markers, for the most part capsaicin-sensitive neurons are peptidergic, small-diameter sensory neurons with unmyelinated C-fibers. These neurons transmit nociceptive information to the central nervous system, whereas their peripheral terminals are sites of release for a variety of proinflammatory mediators [14].

The receptor (VR1) for capsaicin and related vanilloids has been cloned; and it is a ligand-gated cation channel, distantly related to the TRP (transient release potential) proteins, that can also be activated by heat and protons [4,28]. These different classes of activators function coordinately [28]; therefore, VR1 has been suggested to be a molecular ''integrator'' of nociceptive stimuli. After their initial activation of VR1, capsaicin and related vanilloids desensitize VR1 to subsequent stimuli. Although desensitization, the long-term refractory state of the primary dorsal root ganglion neuron following vanilloid exposure, is not fully understood at the molecular level, it represents a potential approach to produce analgesia to subsequent nociceptive challenge.

Resiniferatoxin (RTX), a naturally occurring diterpene derived from the plant *Euphorbia resinifera*, is an ultrapotent analog of capsaicin [6,13,23,27]. RTX binds to VR1 and shows much higher potency both to activate and to desensitize primary DRG neurons than does capsaicin. Moreover, in vivo studies suggest that RTX is relatively more effective for inducing desensitization than for inducing acute responses such as pain; it thus has a more favorable therapeutic profile as compared to capsaicin [23,25]. The numerous in vivo studies with capsaicin,

* Corresponding author. Fax: +1-301-496-8709

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including clinical trials with topical capsaicin treatment, highlight potential therapeutic applications for RTX [8,19,20,30,32]. In accord with the better therapeutic profile for RTX as suggested by the animal studies, the initial clinical trials with RTX for bladder hyperreflexia support its improved selectivity [5,15].

To reduce side effects associated with systemic desensitization of C-fiber pathways by vanilloids, attention has focused on various routes for topical or otherwise localized application. For regional pain, intrathecal or epidural administration represents a strategy for localizing the site of action [8,20,30]. Thus, epidurally administered analgesics are used for the management of chronic tumor pain presenting as ''pelvic pain'' in patients with gynecological, colorectal or genitourinary cancer who experience poor pain control due either to tumor progression or to untoward side effects [17,21]. Well-defined pharmacological advantages of epidural administration include anatomical proximity to the targeted tissue and low systemic uptake.

Although the effectiveness of intrathecal capsaicin remains unresolved in animal models [20,30], epidural capsaicin has been found to produce profound, long-lasting segmental thermal analgesia in the rat [8]. In the present study, we have compared the efficacy of epidurally administered RTX with that administered subcutaneously. Our data demonstrate that epidurally administered RTX produces profound, long-term, segmental thermal analgesia in the rat.

2. Materials and methods

[^{3}H]RTX (37Ci/mmol) was synthesized by the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC. Nonradioactive RTX was from LC Laboratories (Woburn, MA). Male Sprague–Dawley rats (200 g) with an indwelling epidural catheter (L4 level) were purchased from Charles River Laboratories. The plantar analgesia instrument used for the behavioral tests was from Stoelting (Wood Dale, IL).

2.1. Catheter placement

Catheter placement was performed as described by Eisele et al. [9] and by Durant and Yaksh [7]. Using sterile technique, a 1-to 2-cm incision was made over the dorsal vertebral spines of L3–L5, and the superficial fascia and longitudinal paraspinous muscles were bent back laterally from the dorsal processes of L3 and L4. The yellow ligament under the cranial edge of L4 was weakened with a 22-gauge spinal needle with the tip bent at a 90° angle. A 22-gauge polyurethane catheter (Braintree Scientific, Braintree, MA) was introduced under the cranial edge of L4 into the gap created by the spinal needle and was advanced approximately 1–1.5 cm caudally into the epidural space. After satisfactory placement, the catheter was capped and flushed with heparinized saline. Then the catheter was buried subcutaneously and the incision was closed.

2.2. Assay of paw withdrawal latency

The animal protocol was approved by the Animal Care and Use Subcommittee, National Cancer Institute. Animals were kept individually and were allowed access to food and water ad libitum throughout the course of the experiment. Before the experiments, animals were examined to verify that they showed no neurological deficits as a result of the indwelling catheter. The assay for nociceptive response, measuring latency time for paw withdrawal after a radiant thermal stimulus was applied to the paw, was basically as has been described using a plantar analgesia instrument [12]. This approach permitted measurement of paw withdrawal latency both for the back paws as well as for the front paws, thereby providing an internal control for regional change in response in the case of the epidurally administered RTX.

For the measurements, individual rats were placed into the plantar analgesia instrument in a clear plastic chamber with a glass floor. Animals were allowed to acclimatize to their environment for 5 min before measurement commenced. Then a radiant heat source (I.R. Intensity: 50) was positioned under the glass floor directly beneath the paw being evaluated. The radiant heat source and an electronic timer were activated simultaneously. The response of paw withdrawal was detected automatically and the latency was determined to the nearest 0.1 s. To prevent thermal injury, an exposure cutoff of 35 s was used in case of lack of response. Under these conditions visible tissue damage was not observed.

For epidural treatment, the epidural catheter was injected with 40 μl of RTX solution or vehicle control followed by flush of the catheter dead space with an additional 10 μl saline to ensure complete delivery of the drug as suggested previously [7]. The doses of RTX administered epidurally were 1, 3, 10, 30, and 100 μg/kg dissolved in vehicle comprising 10% ethanol, 10% Tween 80, and 80% 0.15 M sterile saline. Hamilton HPLC injector syringes (25–100 μl, 22-gauge needle, Phenomenex (Torrance, CA)) were used for the injections. In the subcutaneously treated groups, doses of RTX ranging from 3 to 300 μg/kg were administered between the shoulder blades in the same vehicle using an injection volume of 250 μl. In all cases, the RTX or vehicle solutions were prepared and administered under sterile conditions. During administration, the animals were briefly anesthetized (60–90 s) using oxygen/isoflurane.

Baseline determination of the latency time for paw withdrawal was carried out 10 min before the administra-

tion of the test dose. Animals were examined to ascertain lack of neurological deficit. Six rats at each RTX concentration in each of two independent experiments were tested for both the epidural and subcutaneous routes of administration, whereas 6 animals served as vehicle treated controls in each experiment. To determine the time-course of desensitization, measurements were carried out on both back and front paws at sequential 30 min intervals for the first 6 h, then at 9, 12, 20 and 24 h, and then daily until the seventh day. The animals were then euthanized. Before the catheterized animals were euthanized, 20 μl methylene blue solution was injected into the catheter to assess its position. 5 rats were excluded from the study because of an improper catheter position. Data are expressed as mean ± S.E.M. Statistical analysis of the data was performed by using a bidirectional analysis of variance (ANOVA) test followed by an unpaired *t*-test where indicated. The criterion for significance was $p < 0.05$.

2.3. [^{3}H]RTX binding assay

Levels of vanilloid receptors in spinal cord membranes were assessed by [^{3}H]RTX binding. Control and treated animals were euthanized by decapitation under CO_2 anesthesia and both the lumbar and cervical segments of the whole spinal cord were removed separately. Membrane preparations from the spinal cord segments were prepared as described [24]. Briefly, samples were disrupted with the aid of an Omni 2000 tissue homogenizer in ice-cold 10 mM HEPES, pH 7.4, containing 5 mM KCl, 5.8 mM NaCl, 2 mM $MgCl_2$, 0.75 mM $CaCl_2$, 12 mM D-glucose, and 137 mM sucrose (buffer A). Homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C; pellets were resuspended in Buffer A and recentrifuged at $35{,}000 \times g$ for 40 min at 4°C. The pellets from the second centrifugation were resuspended in the same buffer at an approximate protein concentration of 2 mg/ml, quick frozen on dry ice as small aliquots, and stored at −70°C until assayed.

[^{3}H]RTX (200 pM) was incubated in a total volume of 300 μl with 100 μg membrane protein for 60 min at 37°C in Buffer A supplemented with 0.25 mg/ml bovine serum albumin (type V, Sigma). The bovine serum albumin was included to reduce nonspecific adsorption of RTX to surfaces. At the end of the incubation, tubes were chilled on ice and 100 μg α1-acid glycoprotein (Sigma) in a 50 μl volume was added to each tube to reduced nonspecific binding. Bound and free [^{3}H]RTX were then separated by pelleting the membranes by centrifugation at $10{,}000 \times g$ for 15 min at 4°C. The tips of the tubes containing the pellets were cut off, and the bound radioactivity was determined by scintillation counting. Nonspecific binding was determined in the presence of 100 nM nonradioactive RTX. Measurements of binding were determined in triplicate in each experiment, and each experiment was repeated at least two times. Binding was expressed as fmol/mg protein. Protein concentration was determined using the Bio-Rad protein assay according to the manufacturer's protocol (Bio-Rad Laboratories, CA).

3. Results

The objective of this study was to evaluate the ability of lumbar epidural RTX to achieve regional desensitization. Measurement of latency of paw withdrawal in response to

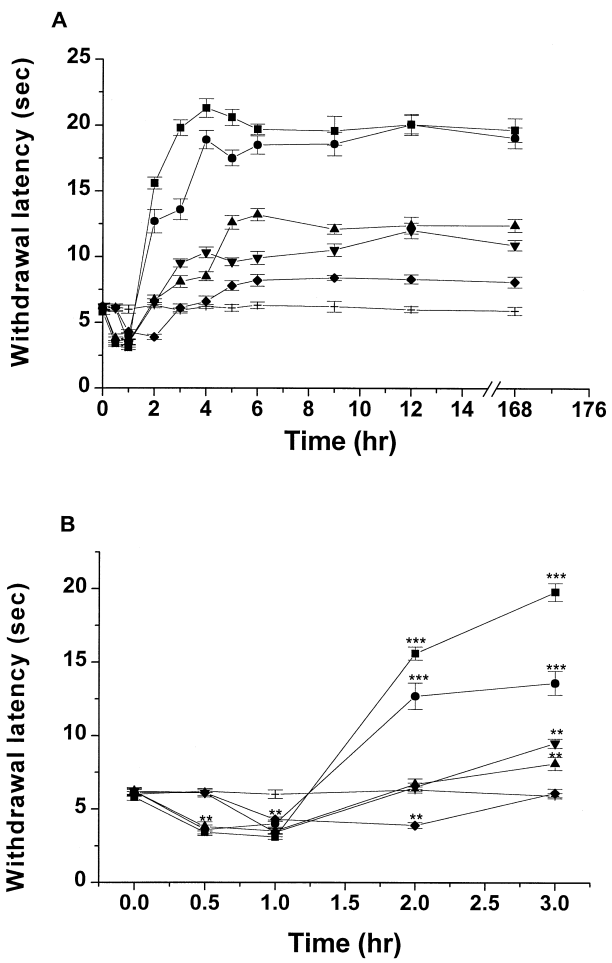

Fig. 1. Time course of the latency of paw withdrawal after treatment with subcutaneous RTX. Animals were treated subcutaneously with vehicle or with 3–300 μg/kg ((+) vehicle, (♦) 3 μg/kg, (▼) 10 μg/kg, (▲) 30 μg/kg, (●) 100 μg/kg, (■) 300 μg/kg) RTX as indicated. Withdrawal latencies of the back paws to thermal stimuli were determined at each time point. Points represent the mean ± S.E.M. ($n = 6$ animals). (A) Time course for the first 168 h after treatment. ANOVA with repeated measures indicated a significant overall difference between RTX- and vehicle-treated groups ($P < 0.0001$) as well as a time-dependent effect ($P < 0.0001$). An unpaired *t*-test revealed a significant increase in latency at each concentration 5 h after treatment compared with the control (vehicle) ($P < 0.001$). (B) Expanded view of panel A for the first 3 h after treatment. Individual comparisons between RTX-and vehicle-treated groups were with an unpaired *t*-test, $^{**}P < 0.05$ and $^{***}P < 0.001$. A second experiment yielded similar results.

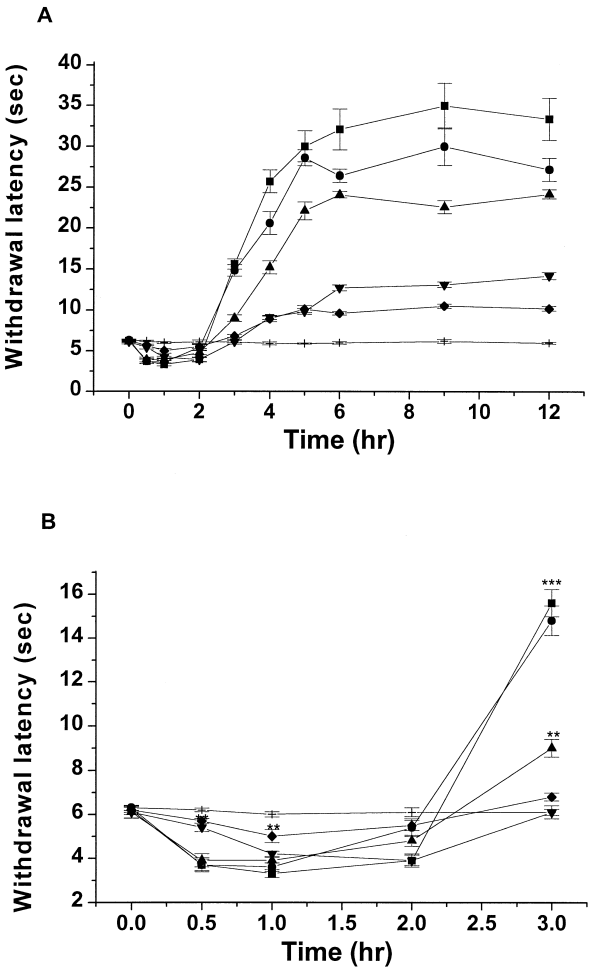

Fig. 2. Time course of the latency of paw withdrawal after treatment with epidural RTX. Animals were treated epidurally with vehicle or with 1–100 μg/kg ((+) vehicle, (♦) 1 μg/kg, (▼) 3 μg/kg, (▲) 10 μg/kg, (●) 30 μg/kg, (■) 100 μg/kg) RTX as indicated. Withdrawal latencies of the back paws to thermal stimuli were determined at each time point. Points represent the mean ± S.E.M. ($n = 6$ animals). (A) Time course for the first 168 h after treatment. ANOVA with repeated measures indicated a significant overall difference between RTX-and vehicle-treated groups ($P < 0.0001$), as well as a time-dependent effect ($P < 0.0001$). An unpaired *t*-test revealed a significant increase in latency at each concentration 4 h after treatment compared with the control (vehicle) ($P < 0.0001$). (B) Expanded view of panel A for the first 3 h after treatment. ANOVA and unpaired *t*-test revealed a significant decrease in latency at each concentration 30 min after treatment compared with the control (vehicle) ($^{**}P < 0.05$ and $^{***}P < 0.001$). A second experiment yielded similar results.

a thermal stimulus using the plantar analgesia instrument permitted comparison of the effects of treatment on the back paws vs. the front paws, providing an internal control for regional effect. Comparison of the lumbar epidural treatment with that of systemic (s.c.) treatment provided a second measure of the selectivity provided by the epidural route of administration.

The effect of RTX on paw withdrawal latency was measured as a function of RTX dose and time after administration. For systemic administration, the paw latency time increased as a function of time after administration, reaching a plateau at 4–5 h (Fig. 1A). Latency values then remained constant for the remainder of the experiment. The onset of increased paw withdrawal latency occurred more rapidly at the higher RTX doses. This difference was most evident at the 2 h time point. The maximal increase in withdrawal latency was to 19.7 ± 0.6 s (average of two experiments) at 300 μg/kg RTX. At this dose, 2 animals died, so higher doses were not examined. The ED_{50} for systemically administered RTX, determined at 6 h, was 39.2 ± 3.9 μg/kg RTX as an average of two experiments (Fig. 3). The slope of the curve was consistent with modest cooperativity, with a Hill coefficient of 1.38 ± 0.26 as the average of two experiments.

The increase in withdrawal latency upon systemic RTX administration was preceded by a period of hyperalgesia, as reflected in a reduction in withdrawal latency (Fig. 1B). The duration of hyperalgesia was more extended at lower doses of RTX and its onset was delayed at the lowest dose examined, 3 μg/kg RTX.

For lumbar epidural RTX administration, the back paw latency time increased to its plateau value modestly more slowly than was the case for systemic administration (Fig. 2A). The plateau latency value was only achieved at approximately 6 h. The values then remained constant for the remainder of the experiment. Unlike for the systemic route of administration, for epidural RTX the onset of increased paw withdrawal latency was similar over the range of RTX doses examined. The maximal increase in withdrawal latency was to 33.4 ± 2.6 s (average of two experiments) at 100 μg/kg RTX. This increase in paw withdrawal latency was significantly greater than that

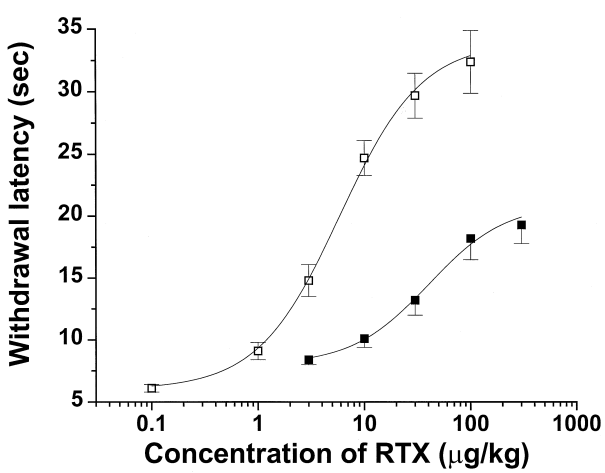

Fig. 3. Dose response of the latency of back paw withdrawal after treatment with epidural (□) or subcutaneous (■) RTX. Withdrawal latencies of back paws were determined 6 h after treatment with epidural or subcutaneous RTX or vehicle control. Points represent the mean ± S.E.M. ($n = 6$ animals). The ED_{50} value for epidural RTX was 5.9 ± 0.3 μg/kg in this experiment. The ED_{50} for subcutaneous RTX was 40.3 ± 3.6 μg/kg in this experiment. A second experiment yielded similar results.

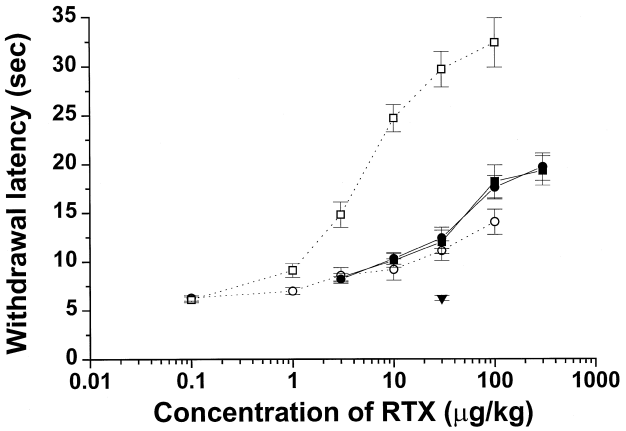

Fig. 4. Comparison of front and back paws for dose response of the latency of paw withdrawal. Withdrawal latencies (solid lines represent the s.c. treated group, (●) front paw and (■) back paws; scattered lines represent the epidurally treated group, (○) front paws and (□) back paws; (▼) control) were determined 6 h after treatment with epidural or subcutaneous RTX or vehicle control. Points represent the mean ± S.E.M. ($n = 6$ animals). Data for hind paws are from Fig. 3. A second experiment yielded similar results.

achievable with the systemic route of administration ($p < 0.001$). Moreover, since we used a 35 s cut off to avoid thermal injury to the animals, it should be noted that this difference might be even greater than the measurements indicated.

The ED_{50} for epidurally administered RTX, determined at 6 h, was 6.0 ± 0.3 μg/kg RTX as an average value of two experiments (Fig. 3). The slope of the curve was consistent with modest cooperativity, with a Hill coefficient of 1.22 ± 0.10. Similar dose response relations were found for the other time points once the plateau in level of desensitization had been achieved, viz. beyond 6 h.

The response of the front paws to lumbar epidural RTX treatment was monitored along with that of the back paws. The dose response curve for the front paws upon lumbar epidural RTX treatment was similar to or less than that observed for either the back or front paws upon systemic RTX treatment (Fig. 4). The systemic effect of the epidural RTX administration thus stands in marked contrast with its significantly enhanced localized effect as evident on the back paws. On the other hand, for systemically administered RTX, little difference was observed in the dose response curves for the front or back paws (Fig. 4), controlling for a difference in intrinsic sensitivity.

For the epidural route of administration, as for the systemic route, the increase in paw withdrawal latency was preceded by a period of hyperalgesia, as reflected in a decrease in withdrawal latency (Fig. 2B). Maximum hyperalgesia was observed at 1 h and, at all concentrations examined, latencies had returned to or beyond baseline by 3 h.

Previously, we have reported that desensitization to systemic RTX administration was associated with loss of vanilloid receptors, as quantitated by binding with [^{3}H]RTX binding [11,22]. To further assess the localization of the effect of epidurally administered RTX, we compared the

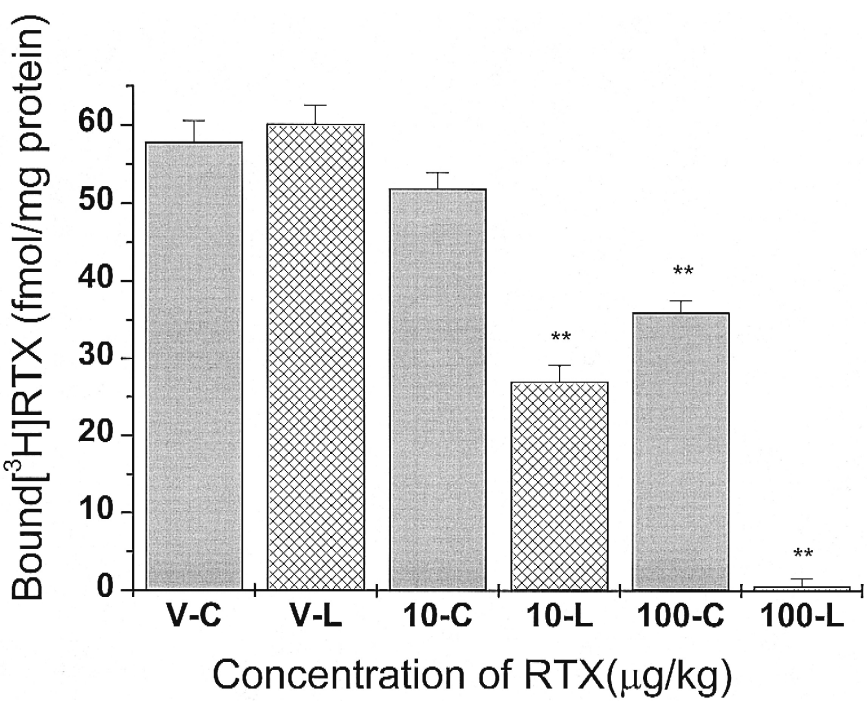

Fig. 5. Specific [^{3}H]RTX binding to membrane preparations of spinal cord obtained from rats treated epidurally with vehicle (V) or with RTX as doses of 10 and 100 μg/kg. Specific [^{3}H]RTX binding was determined at a concentration of 200 pM [^{3}H]RTX to both lumbar (L) and cervical (C) portions of the spinal cord. Values are mean ± S.E.M. ($n = 3$). (**, significantly different from vehicle control, $P < 0.05$.) Two additional experiments gave similar results.

relative levels of [^{3}H]RTX binding to portions of spinal cord removed from the lumbar or thoracic regions 7 days after treatment (Fig. 5). Markedly greater loss of binding was observed for the lumbar region at both the 10 μg/kg and 100 μg/kg doses of epidurally administered RTX. At the 100 μg/kg dose of epidurally administered RTX, 42% loss of specific [^{3}H]RTX binding was observed for thoracic spinal cord compared to the control value. Consistent with this finding, this dose of epidurally administered RTX was sufficient to increase the thermal nociceptive threshold measured on the front paws (Fig. 4).

4. Discussion

The results described here confirm that epidurally administered RTX shows enhanced effectiveness relative to systemic RTX for blocking C-fiber mediated nociception, as measured by latency of paw withdrawal in response to a thermal stimulus. Thermal analgesia is a clinically relevant way of measuring C-fiber mediated nociceptive pain [1,10,18,31]. The enhanced effectiveness is reflected in two parameters, a greater increase in the latency of withdrawal (i.e., greater efficacy) and a shift in the dose response curve to lower doses of RTX (i.e., greater potency). Comparison of the ED_{50} values suggests a 6.5-fold enhancement of potency via the epidural route. An epidural dose of 10% of the systemic dose is often used in clinical practice [17]. Comparison of doses causing comparable 3-fold increases in withdrawal latency indicates a 25-fold enhancement via the epidural route. In the case of epidural capsaicin treatment, using an increase in thermal threshold as the endpoint, an approximately 10-fold enhancement in activity was observed [8].

As was expected, the epidural route of administration provided an enhanced localized effect but did not prevent the gradual systemic absorption of the drug (particularly evident at the highest concentration), which resulted in partial loss of [^{3}H]RTX binding by the cervical region, parallel with a moderate increase in thermal nociceptive threshold on the front paws. Thus, the dose response curve for epidural RTX, evaluated on the front paws, was only modestly shifted relative to that for systemic RTX. Comparison of the response of the front and back paws to systemic RTX provides a control to verify that the front and back paws do not differ in their intrinsic sensitivity.

Resiniferatoxin shows both qualitative as well as quantitative advantages relative to capsaicin as a potential therapeutic agent [26]. In the rat, it is only slightly more potent than capsaicin for inducing chemogenic pain in the eye wiping assay, whereas it is much more potent than capsaicin for desensitizing to chemogenic pain [23]. Capsaicin activates the pulmonary chemoreflex, whereas RTX desensitizes it without prior activation [27]. Capsaicin has been reported to affect molecular targets other than VR1 at concentrations not far removed from those that affect VR1; RTX is more selective as might be expected from its higher potency [23,27]. Perhaps underlying some of the differences in physiological behavior of RTX and capsaicin is that RTX stimulates inward ion currents with markedly slower kinetics than does capsaicin [16,29]. Consistent with the findings in model systems, in the human clinical trials for bladder hyperreflexia intravesicular RTX does not induce pain, unlike capsaicin [5,15]. We suggest that complementing the pharmacological advantages of RTX with regional localization through epidural administration should further enhance its specificity in the treatment of pelvic pain.

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